The Use of Anti-Steroid Antibodies in the Characterization of Steroid Receptors*

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SUMMARY

Antibodies against steroid hormones can be used to eliminate steroid binding to serum proteins and to cellular proteins which are normally not considered to be steroid receptors. Since binding of sex steroids and of some glucocorticoids to their cellular receptor proteins does not appear to be affected by the antibodies, the latter may be used for qualitative and quantitative characterization of the steroid-receptor complexes. The assay method may be simplified by the use of anti-steroid antibodies coupled to solid phases such as Sepharose. A large number of small samples can be processed within 3 to 4 hours. Samples containing about 1 to 5 fmol of receptor-bound ³H steroids may be assayed.

Studies made during the last decade have provided strong evidence that steroid hormones act by binding to specific proteins in the target cells (1-6). These cellular receptors for steroid hormones are generally identified by their high affinity toward specific groups of active steroids.

Qualitative and quantitative analyses of the receptor proteins are often complicated by the presence of other tissue and blood proteins that bind the same steroids tightly, but nonspecifically. One of the most useful techniques for distinguishing specific steroid-receptor complexes from nonspecific steroid binders has been to identify the 8 S form in gradient media of low ionic strength (5). The steroid receptors are known to be present in multiple forms, however, and the amount of the 8 S form present does not always indicate or reflect the total receptor content. In crude cellular extracts the 8 S form is an unstable entity that does not always indicate or reflect the total receptor content. Multiple forms, however, and the amount of the 8 S form present can be processed within 3 to 4 hours. Samples containing about 1 to 5 fmol of receptor-bound ³H steroids may be assayed.

Experimental Procedure

Materials—The radioactive steroids used, all of which were obtained from New England Nuclear Corp., were [1,2-³H]testosterone (44 Ci per mmol), 5α-[1,2-³H]dihydrotestosterone (44 Ci per mmol), [1,2-³H]cortisol (46 Ci per mmol), 17β-[2,4,6,7-³H]estradiol (106 Ci per mmol), [1,2-³H]dexamethasone (88 Ci per mmol), and [1,2-³H]progesterone (48 Ci per mmol). Anti-steroid antibodies were purchased from Research Plus Inc. or Endocrine Sciences. These antibodies were produced by immunization of rabbits or sheep with bovine serum albumin conjugated to testosterone-3-(O-carboxymethyl)oxime, estradiol-17β-succinate, progesterone-20-(O-carboxymethyl)oxime, or cortisol-21-hemisuccinate. The resulting antisera was treated with bovine serum albumin to remove antibodies directed against the carrier protein.

Preparation of Tissue Extracts and ³H Steroid-Receptor Complexes—The fresh tissue samples from the animals were minced and homogenized in 2 volumes of 20 mM Tris-HCl buffer, pH 7.5, containing 1.5 mM EDTA, in an all glass Potter-Elvehjem homogenizer at 0-2°C. The radioactive steroid was added to the homogenate (0.5 PCi per ml). Addition of steroids at this stage usually gave higher yields of ³H steroid-receptor complexes than if steroids were supplied later, apparently because the steroids protect the receptors from degradation (3, 4). The mixture was incubated at 0°C for at least 1 hour before further manipulations were carried out.

A long incubation is generally needed for adequate labeling of the receptor proteins with the radioactive hormones, since the rate of association is a slow process at the low temperature employed (3, 4). After incubation, the mixture was centrifuged at 600 × g for 10 min in a Sorvall centrifuge with a SS 34 rotor. The supernatant was then centrifuged at 100,000 × g for 60 minutes to yield the cytosol fraction. The protein contents of the resulting supernatant were 1.5-2.5 mg in 0.5 ml. Samples containing about 1 to 5 fmol of receptor-bound ³H steroids may be assayed.

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tional Research Fellowship.
the cytosol preparations were about 20 mg per ml for rat liver and ventral prostate and 3 to 5 mg for rat uterus.

In some experiments, partial purification of the $^3$H steroid-receptor complexes was achieved through precipitation of the complexes by the addition to the cytosol mixture of ammonium sulfate to 40% saturation with respect to the salt. The precipitate was dissolved in the Tris-EDTA buffer mentioned above and dialyzed against phosphate buffer (pH 7.5) through a Sephadex G-25 column. The sedimentation technique effectively eliminated the non-steroid proteins that also bind the radioactive steroids (13). The amounts of $\alpha$-[$^3$H]dihydrotestosterone bound to receptors in the fractionated prostate preparations were about 15,000 cpm per mg of protein.

If whole tissue samples are to be analyzed, it is convenient to homogenize them in 2 volumes of 0.6 M KCl solution containing the Tris-EDTA buffer described above and the required $^3$H steroid. The homogenate is then allowed to stand at $0^\circ$ for 1 hour, centrifuged, and the extract used for analysis.

**Antibody Preparation** The commercially available antibodies (1 to 2 mg of protein per ml) used for the steroid assay were found to be satisfactory without further purification. Antibodies with low protein concentration were as effective as those that were highly specific. The capacity of the antibody preparation used must be sufficiently large for quantitative removal of 95% or more of the $^3$H steroid added to the assay tubes. In the assay methods described below, the use of antibodies in large excess is not harmful.

For the preparation of insoluble antibodies, the antibodies are precipitated from the commercially supplied solution by the addition of $\text{Na}_2\text{SO}_4$ (0.1 or 0.2 mg per ml). The antibody precipitate (for the lyophilized antibody supplied commercially) is washed twice with 2.5 ml of 18% (w/v) $\text{Na}_2\text{SO}_4$. The sediment (1 to 2 mg of protein) is then suspended in 1 ml of 0.1 M $\text{NaHCO}_3$ containing 0.5 M NaCl. The suspension can be stored at $-20^\circ$ if it is to be used on the same day. The following procedure, adapted from that described by other workers (14, 15), has been found to be satisfactory for the coupling of anti-steroid antibody to Sepharose. Cyanogen bromide (CNBr)-activated Sepharose, 200 mg, is swollen and washed in a glass filter with 1 mM HCl (40 ml) for 15 to 30 min at room temperature. The washed CNBr-Sepharose is then suspended in 4 ml of 0.1 M $\text{NaHCO}_3$ containing 0.5 M NaCl and mixed with 1 ml of the antibodies prepared as described above. The mixture is then centrifuged in a clinical centrifuge, and the gel sediment is reacted with 1 mM ethanolamine (pH 8) for 1 hour at $0^\circ$ to eliminate any remaining active groups. The gel is washed three times with 0.1 M acetate buffer containing 1 M NaCl at pH 4, followed by three washings with 0.1 M borate buffer containing 1 M NaCl at pH 8. The washed gel is suspended in 2 ml of 20 mM Tris-Cl buffer containing 1.5 mM EDTA at pH 7.5, and stored at $-20^\circ$. About 90% of the antibodies added can be coupled to Sepharose by this procedure.

**Receptor Assay Method I (with Gradient Centrifugation)**—All manipulations are carried out at $0^\circ$ to $4^\circ$. The sample solution containing $^3$H steroid-receptor complexes (0.05 to 0.2 ml) is mixed with a solution (0.05 to 0.2 ml) of antibody that has the capacity to bind essentially all the $^3$H steroid present. The final concentration of KCl is made to 0.4 M by the addition of 2 M KCl. After the mixture has been allowed to stand for 2 hours, a portion of the sample (0.1 or 0.2 ml) is layered on the top of a sucrose (5 to 20%) gradient medium containing 20 mM Tris-Cl buffer, 0.4 M KCl, and 1.5 M EDTA, pH 7.5. The tube is centrifuged at 54,000 rpm for 18 hours in a Beckman-Spinco L2-65B ultracentrifuge with a SW 50 rotor. After centrifugation, the fractions can be collected either from the top or from the bottom of the tube and the radioactivity of each fraction measured by a liquid scintillation spectrometer (13).

**Receptor Assay Method II (with Solid Phase Antibody)—Incubation of the sample is carried out in the same way as in Method I, except that antibody coupled to a solid phase such as Sepharose is used. The mixture (in a centrifuge tube) is stirred for 2 hours and then centrifuged at 3000 rpm for 10 min in a clinical centrifuge. An aliquot of the supernatant is taken for the measurement of radioactivity.**

Control tubes for the measurement of background radioactivity can be set up by omitting the biological sample (but using the same quantity of the radioactive steroid as in the experimental tubes), or by heating of the sample before the addition of steroids. Heating of the tissue sample in the presence of a radioactive steroid, however, often results in a high background that interferes with the accurate measurement of the receptor content.

**Other Methods**—Human serum obtained from fresh blood was dialyzed overnight against a buffer containing 1.5 mM EDTA and 20 mM Tris-Cl, pH 7.5, at 0 to $2^\circ$ to remove the endogenous steroids. Protein content is determined by the method of Lowry et al. (16), with bovine serum albumin as the standard. Radioactivity was measured in a Packard scintillation counter; the scintillation fluid being a mixture of 1.5 liters of toluene, 500 ml of Triton X-100, 8 g of 2,5-diphenyloxazole, and 100 mg of 1,4-bis[2(5-phenyl oxazoyl)]benzene. The counting efficiency was 60%.

**RESULTS**

**Effect of Anti-Steroid Antibodies on Sedimentation Patterns of Prostate Cytosol Proteins Labeled with $\alpha$-[$^3$H]Dihydrotestosterone**

Fig. 1A shows the sedimentation patterns of $\alpha$-[$^3$H]dihydrotestosterone associated with a cytosol preparation of rat ventral prostate. In the absence of the antibody to the androgen, the protein-bound radioactive steroid migrated as a single peak at about 3 to 4 S in the gradient solution containing 0.4 M KCl (13). The steroid not bound to proteins stayed near the top of the centrifuge tube. If antibody was added, essentially all of the free steroid as well as a large portion of the 3 to 4 S radioactivity was removed. A concomitant 8 S peak appeared which showed the binding of $^3$H steroid by the antibodies.

As the amount of antibody was increased, the 3 to 4 S peak reached a minimum level (Fig. 2). Other experiments (see below) showed that the antibody-resistant levels were linearly proportional to the quantity of cytosol used.

**Evidence that Receptor-bound $\alpha$-[$^3$H]Dihydrotestosterone Is Not Affected by Anti-Steroid Antibody If $\alpha$-[$^3$H]dihydrotestosterone...**

![Diagram](https://example.com/diagram.png)

**FIG. 1. Effect of an anti-androgen antibody (Ab) on the sedimentation pattern of $\alpha$-[$^3$H]dihydrotestosterone ($^3$H/DHT) bound to the cytosol proteins of rat ventral prostate or to the isolated prostate androgen receptor.** In the experiment, the results of which are shown in A, 0.4 ml of prostate cytosol containing 12,000 cpm of the radioactive androgen was incubated in a final volume of 0.5 ml alone (C) or in the presence of anti-$\alpha$-dihydrotestosterone (○) to antibody (20 µg of protein). In the experiment shown in B, $\alpha$-[$^3$H]dihydrotestosterone receptor complex (4000 cpm/0.78 mg of protein) isolated from rat ventral prostate was incubated alone (C) or in the presence of the same antibody (○). The incubated mixtures were analyzed by gradient centrifugation. The detailed procedures are the same as those described under ‘‘Experimental Procedure,’’ except that, in B, the gradient medium contained 10 to 30% sucrose. Gradient centrifugation was carried out for 18 hours (A) or 16 hours (B). Bovine albumin (4.6 S) was collected in Fractions 7 and 8, respectively, in A and B.
receptor complex was first separated from the nonspecific steroid-binding protein in the prostate cytosol and then analyzed by gradient centrifugation, the level of the 3 to 4 S radioactivity peak (due to the steroid-receptor complex) remained the same in the absence and in the presence of the antibody (Fig. 1B). Other experiments also showed that the nonspecific steroid binders in the α protein fraction (13) of the prostate cytosol lost all of the steroid if antibody was present. These results indicated that the antibody-resistant 3 to 4 S peak essentially was due to the specific androgen-receptor complex.

This conclusion is supported by a study of the steroid specificity. In this study, the radioactive 5α-[3H]dihydrotestosterone, testosterone, progesterone, and cortisol were each incubated with the prostate cytosol and analyzed. The result indicated that H steroids bound to the 3 to 4 S proteins nonspecifically to some extent if no antibody was present. In the presence of the individual anti-steroid antibodies, a clear 3 to 4 S antibody-resistant peak was observed with the cytosol containing 5α-[3H]dihydrotestosterone, but not with the cytosol containing [3H]progesterone (Fig. 3B) or [3H]cortisol (result not shown). [3H]-Testosterone also gave a small but definite antibody-resistant profile (Fig. 3A), confirming the earlier data showing weak binding of testosterone to the androgen receptor of rat ventral prostate (Refs. 3 and 15 and reports cited therein).

Fig. 4A shows the result of an experiment in which rat liver cytosol labeled with [3H]cortisol was used. Since cortisol could bind to a glucocorticoid receptor and to two or three other proteins in the cytosol (17, 18), the antibody-resistant 3 to 4 S peak constituted only a small portion of the total protein-bound steroid. If [3H]dexamethasone, which binds essentially to the receptor protein only (6, 17, 18) was used, more than 90% of the 3 to 4 S binding was antibody-resistant. In the presence of the antibody, the hepatic receptor preparation bound far fewer [3H]-cortisol (B) was incubated alone (O) or in the presence (●) of an fast antibody (20 μg of protein) against testosterone (left) or progesterone, respectively. After incubation, 0.2 ml of each sample was analyzed by gradient centrifugation. A similar experiment with [3H]cortisol showed that there was very little antibody-resistant protein binding of [3H]cortisol in the prostate cytosol.

Fig. 5. Effect of anti-glucocorticoid antibody (Ab) on binding of [3H]testosterone and [3H]progesterone by the cytosol proteins of rat ventral prostate. The experiments were carried out in the same way as described in Fig. 1A, except that 0.4 ml of cytosol containing 12,000 cpm of [3H]testosterone (A) or 0.2 ml of cytosol containing 2,000 cpm of [3H]progesterone (B) was incubated in a final volume of 0.5 ml alone (O) or in the presence (●) of an antibody (20 μg of protein) against testosterone (left) or progesterone, respectively. After incubation, 0.2 ml of each sample was analyzed by gradient centrifugation.
Measurement of \[^{3}H\]estradiol-receptor complex of rat uterine cytosol in presence of \[^{3}H\]cortisol

Table I: Measurement of \[^{3}H\]estradiol-receptor complex of rat uterine cytosol in presence of \[^{3}H\]cortisol

<table>
<thead>
<tr>
<th>Radioactive steroid</th>
<th>Antibody against</th>
<th>Radioactivity associated with 3 to 5 S complex</th>
</tr>
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<tbody>
<tr>
<td>[^{3}H]estradiol</td>
<td>[^{3}H]cortisol</td>
<td>Estradiol</td>
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<tr>
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<td>+</td>
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Fig. 5. Effect of anti-steroid antibodies (Ab) on the binding of radioactive steroids by human serum proteins. The serum, obtained from a 26-year-old normal female, was diluted 80 times with a buffer containing 1.5 mM EDTA and 20 mM Tris-HCl buffer, pH 7.5, and mixed with radioactive 5α-dihydrotestosterone (\[^{3}H\]DHT), progesterone, or cortisol. The mixture (0.4 ml) was incubated with 1400, 640, or 1600 cpm, respectively, for 2 more hours. The mixture was then analyzed by gradient centrifugation, and the radioactivity associated with the 3 to 5 S peak was measured. Details are described under “Experimental Procedure.”

Table I shows the results of an assay of estradiol-receptor complex in a uterine cytosol preparation. Since this tissue contains a very small quantity of glucocorticoid receptor compared to the latter could be measured even in the presence of a mixture of \[^{3}H\]estradiol and \[^{3}H\]cortisol if antibodies to both estradiol and cortisol were added.

Elimination of Steroid Binding due to Blood Contamination—

The detection and quantitative measurement of the steroid receptors are often complicated by blood contamination, since the blood of certain animals and humans contains several steroid-binding proteins (3, 20). Fig. 5 shows that such contamination may be eliminated by use of the appropriate anti-steroid antibodies. In this experiment, binding of radioactive 5α-dihydrotestosterone, progesterone, and cortisol to the human serum proteins was shown by gradient centrifugation. If an antibody against the individual steroid was added to the serum, such binding was completely abolished.

Use of Insolubilized Antibody in Characterization of Cellular Steroid Receptors—If the anti-steroid antibody is fixed to an insoluble material and then employed in the above experiments, one can estimate antibody-resistant steroid binding by measuring the radioactivity of the supernatant after a brief centrifugation of the mixture in a low speed centrifuge. The result of one such experiment is shown in Fig. 6. The anti-5α-dihydrotestosterone antibody coupled to Sepharose was incubated with a prostate preparation labeled with 5α-[\(^{3}H\)]dihydrotestosterone. The radioactive androgen bound to the antibody was then removed by centrifugation, whereas the androgen remaining in the solution was measured. The results show that the antibody-resistant binding can be estimated easily by this method. When the soluble fraction was analyzed by gradient centrifugation, we found that essentially all of the antibody-resistant radioactivity was in the 3 to 4 S region. The gradient centrifugation study of the fractionated 5α-[\(^{3}H\)]dihydrotestosterone receptor complex of ventral prostate (Fig. 7) also indicated that the free steroid was indeed removed by the Sepharose-bound antibody, but that the receptor-bound \[^{3}H\]androgen remained intact in the solution.

We have employed this technique to measure the receptor content in the cytosol fractions of several target tissues of steroid hormones. Fig. 8 shows that the cytosol receptors for estrogen in the rat uterus and for androgen in the rat ventral prostate can be determined quantitatively with this new method. Our other experiments also showed that the receptor content of a whole tissue, of cell nuclei, or of other cellular particulates can be measured if the particulate samples are first extracted with...
0.4 M KC1 (see “Experimental Procedure”) and if the extract is
needed to remove the particulate materials by ultracentrifugation.

and the amounts of protein in the cytosol used, on the abscissa.
radioactivity (receptor-bound steroid) are shown on the ordinate

with the 4 to 5 S protein. Details of the method are described
under “Experimental Procedure.” For the measurement of the
androgen receptor in the prostate cytosol (24 mg of protein per
ml) (B), the experiment was carried out in the same manner as
in the Experiment A except that the prostate cytosol was omitted.

Steroid antibody complex can also be removed from the solution
by the addition of a second antibody that precipitates the steroid-
receptor complex (Fig. 9A). We found that this technique was
useful for the analysis of samples having a high steroid-receptor
content. With certain batches of the second antibody, however,
we found that, when 3H steroid, anti-steroid antibody, and a
second antibody were mixed together, a new soluble complex was
formed in the absence of a cytosol preparation (Fig. 9B). Such
a technique should therefore be avoided if the samples contain
low levels of steroid receptors.

**DISCUSSION**

In the methods proposed above for the characterization of
steroid receptors, the samples are prepared and assayed in the
presence of media containing high concentrations of KCl.
Under these conditions, essentially all of the cellular receptors
that bind steroids tightly are known to sediment in the 3 to 5 S
region (1-3). The methods therefore are more appropriate for
the quantitative assay of steroid receptors than that based only
on the existence of 8 S forms. The assay of steroid receptors by
gel filtration or adsorption techniques also presents some com-
plications, often due to the use of excess quantities of the adsorp-
tion media that bind steroids and cellular proteins (including
receptors) rather nonspecifically (7-11). With our new meth-
ods, there is no indication that the accuracy of measurement is
diminished in the presence of excess anti-steroid antibodies.

If a solid phase antibody is employed, the manipulation is
simple, no costly setup is needed, and a large number of small
samples can be processed within 3 to 4 hours. Samples contain-
ing about 1 to 5 fmol of the receptor-bound H steroids can be
measured. For target tissues that are rich in steroid receptors
(30 to 100 fmol per mg of cytosol protein), 10 to 30 mg of tissue
may be sufficient for the assay. If the receptor content is low,
100 to 300 mg of tissue may be required for accurate measure-
ments. The use of a large excess of H steroids should be avoided
so that the background radioactivity can be maintained at a low
level. If necessary, the background radioactivity can be reduced
receptors. This is in marked contrast to steroid-metabolizing enzymes or blood steroid-binding proteins which generally recognize only a portion of the steroid molecule (20, 23). It is possible, therefore, that some of the anti-steroid antibodies can also interact directly with the steroids peripherally bound to the nonreceptor proteins (but not with those bound to the receptor) to facilitate the dissociation of steroids.

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